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<b>(54) Title:</b> PROCESS FOR SEPARATING STEREOISOMERS OF FOLINIC ACID  <b>(57) Abstract</b>  The (6S) stereoisomer of folinic acid is obtained in high optical purity by salification of (R, S) folinic acid with di- or polyamines and subsequent selective crystallization of the desired diastereomeric salt.		

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PROCESS FOR SEPARATING STEREOISOMERS OF FOLINIC ACID

The present invention relates to a process for separating stereoisomers of folinic acid.

Folinic acid, i.e. N-(5-formyl-(6R,S)-5,6,7,8-tetrahydropteroyl)-L-glutamic acid, when obtained by  
5 chemical synthesis, is formed by an equimolar mixture of its two (6R) and (6S) diastereomeric forms.

It is known that only the (6S) isomer, as calcium salt, has the well-known pharmacological activity of the product, while the other one is totally devoid of it.  
10 Therefore, there is a strong need for a process allowing the preparation of the optically pure (6S) form.

Various attempts were made to synthesize (6S) isomer through asymmetrical synthesis but they were not fully  
15 successful from the industrial point of view.

As far as the separation of both diastereomers from their equimolar mixture is concerned, it is worth citing US patent 2688018 and patent application WO 88/03844, even if they have not yet provided the ideal  
20 solution to the problem.

It has now been found a process allowing the separation and the isolation with good yields of the two optically pure (6R) and (6S) forms of folinic acid, both free and salified with alkaline-earth metal ions, from the  
25 equimolar diastereomeric mixture produced by chemical synthesis starting from folic acid.

Another remarkable embodiment of this invention relates to a process for the preparation and isolation of salt derivatives of (6R)-folinic acid, respectively (6S)-

folinic acid, characterised by an excellent optical purity (O.P.), with suitable at least dibasic organic amines, which are aliphatic, straight, cyclic and/or heterocyclic, from said diastereomeric equimolar mixture.

Another remarkable embodiment of this invention is represented by the new salts of (6R)-folinic and/or (6S)-folinic acid with said amines.

Folinic acid is usually prepared by synthesis starting from folic acid, which contains a (S) chiral centre in that part of the molecule corresponding to (S)-glutamic acid. By hydrogenating the double bond between the 5- and 6- positions of the pterinic residue, a new chiral centre in the 6- position is originated with a (6R,S) configuration. The subsequent formylation step of nitrogen atom at the 5- position leads to the production of (6R,S)-folinic acid.

It now has been surprisingly found that, by crystallization of (6R,S)-folinic acid salts with at least dibasic organic amines from a suitable solvent or, preferably, from binary or ternary mixtures of solvents, mixtures of said salts enriched in their (6R) or (6S) form can be obtained, according to the amines used.

Said optically enriched mixtures can be further enriched in the preponderant isomeric form through one or more recrystallizations to obtain the corresponding salts with said amines of (6R)-folinic acid, respectively (6S)-folinic acid, with an O.P. of at least 99%, usually higher than 99%.

The conversion from these salts to the corresponding

salts with alkaline-earth metal ions with the same optical purity is very easy.

By way of example, from the mixture enriched in its (6S)-form, the calcium salt (or the corresponding salts with alkaline-earth metal ions) of (6S)-folinic acid  
5 can be prepared with an O.P. higher than 99%. This result can preferably be achieved according to one of the following methods:

a) from the aqueous solution of the (6S) enriched  
10 diamino folinate, through treatment with an alkaline-earth metal chloride at neutral pH and optionally in presence of a precipitating solvent, the alkaline-earth metal salt of the (6S) enriched folinic acid is separated. Further crystallizations  
15 from a suitable solvent or solvent mixtures lead to the optically pure (6S) form of the desired alkaline-earth metal folinate,

b) the (6S) enriched diamino folinate is  
recrystallized from suitable solvent mixtures in  
20 order to obtain the optically pure (6S) form. The corresponding salt with alkaline-earth metal ions is subsequently obtained through exchange, as described in a).

The same process can be applied to the (6R) enriched  
25 mixture to obtain the corresponding (6R) isomer with an O.P. higher than 99%.

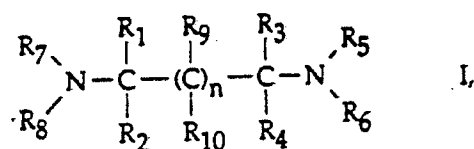
Preferred metal cations to salify (6R,S)-folinic acid and its optically pure diastereomeric forms are those of the alkaline-earth metals, especially Ca, Mg, Sr,  
30 Ba.  $\text{Ca}^{2+}$  ion is particularly preferred.

At least dibasic organic amines suitable for the

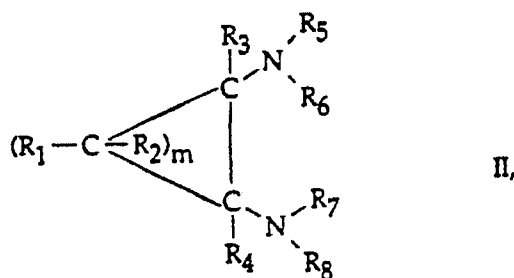
salification of (6R,S)-folinic acid can be selected from straight, cyclic or heterocyclic, substituted or unsubstituted, racemic or chiral, aliphatic di- or polyamines, which contain at least two amino groups  
 5 linked to one another by at least one substituted or unsubstituted hydrocarbon chain, comprising at least 2 or 3 C atoms.

Particularly preferred amines are diamines of general formulae from I to III,

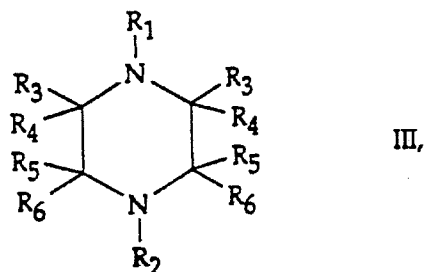
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wherein:

R<sub>1</sub> to R<sub>8</sub> which are the same or different, are H or straight or branched C<sub>1-6</sub> alkyl groups, which can be substituted by 1-4 OH groups and/or by  
 30 1-4 alkoxy or hydroxyalkoxy groups,

R<sub>9</sub> to R<sub>10</sub> which are the same or different, are R<sub>1</sub> or OH,

n 0-6,

m 2-8.

By way of non-limiting example, particularly preferred diamines can be selected from: ethylenediamine, 1,2-  
5 diamino-propane, 1,3-diamino-propane, 1,3-diamino-2-hydroxy-propane, (cis)-1,2-diamino-cyclohexane, (trans)-1,2-diamino-cyclohexane, piperazine, 1,4-dimethyl-piperazine, 2-methyl-piperazine, 2,5-dimethyl-piperazine.

Equally preferred amines can also be polyamino straight  
10 derivatives or macrocyclic compounds containing from 3 to 6 N atoms.

By way of example, among these last ones, 1,4,7,10-tetraazacyclododecane and its derivatives substituted both on ethylene bridges and on nitrogen atoms can be  
15 cited.

Suitable solvents for the crystallization of diastereomeric mixtures of folinic acid salts with said amines are preferably binary or ternary water/dipolar aprotic organic solvent mixtures and/or mixtures of  
20 water/dipolar aprotic organic solvent/protic organic solvent.

Preferred protic organic solvents are, i.e., methanol, ethanol, n-propanol, isopropanol, ethylene glycol, 1,2-propylene glycol, 1,3-propylene glycol, formamide, N-methyl-formamide.  
25

Preferred dipolar aprotic organic solvents are, i.e., dimethylformamide (DMF), dimethylacetamide (DMAC), dimethylsulphoxide (DMSO), N-methyl-pyrrolidone (NMP), hexamethylphosphoramide (HMPT).

30 The process of this invention is hereinafter described, employing, by way of non-limiting example, as starting

material the calcium salt of (6R,S)-folinic acid and it is performed according to the following steps:

#### Step 1

5      Precipitation of (6R,S)-folinic acid from an aqueous solution of its calcium salt, by adjusting pH between 1 and 3 with mineral acids at a temperature ranging from 0 to 25°C.

#### Step 2

10      Formation of (6R,S)-folinic acid salts with diamines/polyamines in a dipolar aprotic organic solvent, preferably DMAC. Said salts are obtained under a solid form by:

- 15      a) dissolution of (6R,S)-folinic acid in a dipolar aprotic solvent, in a weight/volume ratio ranging from 1:5 to 1:20 (w/v);
- 20      b) reaction, at a temperature from 5 to 35°C, with a stoichiometric amount, or a molar excess not higher than 20%, of the desired amine, dissolved either in the same dipolar aprotic solvent in a ratio ranging from 1:5 to 1:50 (w/v) or in a protic organic solvent in a ratio ranging from 1:5 (w/v) to 1:15 (w/v);
- 25      c) gradually cooling the reaction medium at a temperature ranging from 25°C to 5°C, up to a complete precipitation of the desired salt. Precipitation can be eased by adding a protic organic solvent in a volume ratio ranging from 0.1:1 to 2:1 (v/v) compared with the dipolar aprotic solvent.

30      Said salification reaction can also be performed in water, in suspension phase, followed by dilution of the



resulting aqueous solution with or in a water-soluble protic organic solvent, which acts as a precipitating agent.

### Step 3

5 Partial separation of folinic acid diastereomers by crystallization or insolubilization of its salts with said amines from a water/dipolar aprotic water-soluble solvent, optionally with addition of an organic protic solvent.

10 The weight/volume (w/v) ratio salt/mixtures of solvents can range from 1:2 to 1:60, preferably from 1:4 to 1:45.

The composition of the mixture water/dipolar aprotic solvent (v/v) can range from 1:0.5 to 1:20, preferably  
15 from 1:1 to 1:15; such mixture can also contain a protic organic solvent in amounts up to 70% in volume, preferably to 50%, in case said protic organic solvent is needed.

The salts of (6R,S)-folinic acid with the above  
20 mentioned amines can directly be dissolved in the desired mixture of solvents at a temperature comprised between 25 and 70°C, preferably 30 and 50°C.

On the other hand, they can also be dissolved in water. The desired amount of dipolar aprotic solvent,  
25 optionally together with the required additional amount of protic organic solvent, is subsequently added to said aqueous solution.

Then these solutions are gradually cooled to nearly 0°C while crystallization takes place.

30 The obtained solid moiety consists of a mixture of (6R) and (6S) isomers of amino-folinates, which is enriched

in the less soluble form (i.e. R or S, depending on the amino compound used as salifying agent).

For example, crystallization of ethylenediamino (6R,S)-folinate leads to a mixture enriched in the (6R) isomer salt.

On the contrary, from piperazino (6R,S)-folinate, the (6S) form preferably crystallizes.

Usually said enriched solid mixtures show an O.P. ranging from 65 to 98%.

Crystallization mother liquors are obviously enriched in the other isomeric form and show an O.P. value usually ranging from 65 to 80%.

#### Step 4

Further purification of the partially optically purified amino folinates, deriving from Step 3, is performed through one or more recrystallizations of the same from a mixture water/dipolar aprotic solvent whose composition ranges from 1:1 (v/v) to 1:20 (v/v). This crystallizing mixture can optionally contain also a protic organic solvent in amounts up to 50% in volume. The desired optically pure amino folinate is obtained with an O.P. higher than 99%.

Then said optically pure amino folinate can be transformed into the corresponding (6R), respectively (6S), calcium folinate.

One of the preferred ways consists in crystallizing said amino folinate from water (pH = 7 and temperature in the range 0-25°C) in the presence of 0.2 to 5, preferably 2 to 4, parts (w/w) of water soluble calcium salts, preferably chlorides or nitrates.

Alternatively the desired optically pure calcium

folinate can be precipitated from the above mentioned aqueous  $\text{Ca}^{2+}$  containing solution by adding a great excess of a protic water-soluble organic solvent.

#### Step 5

5 The stereoisomers of folinic acid, which are contained in the mother liquors resulting from the crystallizations described in Step 3, are also recovered from said mother liquors with an O.P. higher than 99%.

10 They can be obtained either as amino or calcium salts, preferably following one of the procedures hereinafter described.

a) From the above mentioned mother liquors the organic protic solvent, if present, is distilled  
15 off under reduced pressure at temperatures from 25 to 50°C and subsequently the water-soluble dipolar aprotic solvent is removed by extracting the mixture with a water-insoluble organic solvent, preferably methylene chloride ( $\text{CH}_2\text{Cl}_2$ ). To the  
20 resulting aqueous solution  $\text{CaCl}_2$  is added in amounts corresponding to 0.2-5, preferably to 2-4, parts in weight (w/w) in relation with the starting quantity of amino (6R,S)-folinate. Then pH is adjusted to a value ranging between 6 and 7 by  
25 means of 1N NaOH and, by adding ethanol the precipitation of the desired calcium salt is obtained, operating at a temperature of 0-25°C, preferably 5-25°C.

30 Calcium folinate, enriched in its prevailing diastereomer, with an O.P. ranging from 65 to 85%, is recovered through filtration. The optically pure

calcium folinate (O.P. higher than 99%) is finally obtained through one or more crystallizations from 10 to 100 volume of water, with pH ranging from 6 to 7 and at temperatures from 0 to 25°C.

- 5     b) To mother liquors, deriving from Step 3, suitable precipitating solvents, either dipolar aprotic or protic organic solvents, are added in amounts equal to 0.05-5 volumes in relation with the mother  
10     liquors volume. By cooling to 0-20°C, the prevailing optical isomer of diamino folinate can be preferentially crystallized with an O.P. higher than 70%:

15     The desired amine folinate with an O.P. higher than 99% is then obtained by one or more crystallizations from water/dipolar aprotic solvent mixtures with a ratio ranging from 1:1 to 1:20 (v/v), optionally by adding a protic organic solvent, in amounts up to 50% in volume.

20     Amino (6R,S)-folinate obtained as described in Steps 1 and 2 can alternatively be prepared directly from a calcium (6R,S)-folinate aqueous solution. To this solution another aqueous solution can be added containing, in stoichiometric amounts compared with the starting folinate, the desired amine salified with a  
25     mineral or organic acid which forms insoluble salts with alkaline-earth metal cations, preferably anions of sulphuric or oxalic acid. The insoluble salt precipitating, i.e. calcium oxalate, is filtered off and to the resulting aqueous solution, containing the  
30     desired diamino (6R,S)-folinate, the suitable amount of dipolar aprotic solvent is subsequently added,

optionally added with protic organic solvent as previously described.

Crystallization can be performed at a temperature ranging from 0 to 25°C and lasts from 1 h up to five

5 days by operating at a neutral or slightly alkaline pH.

The procedure described in step 1 can also be adopted in order to obtain the optically pure isomers of free folinic acid (6R or 6S), with an O.P. higher than 99%, starting from the corresponding optically pure salts of

10 the same obtained according to step 4 and 5.

The following examples aim at better disclosing the most remarkable features of this invention, and are not a limit to the same for the skilled technician.

#### EXAMPLE 1

15 Preparation of pure (6R,S)-folinic acid.

100 g of calcium (6R,S)-folinate pentahydrate, dissolved in 750 ml of water and 670 ml of 0.5 N hydrochloric acid, are added dropwise at the same time to 300 ml of water, under stirring and at 5-20°C. Then  
20 the obtained suspension, containing (6R,S)-folinic acid, is kept under stirring for 1 h at 5-25°C. After that the precipitate is filtered, washed with water and dried. 76 g of (6R,S)-folinic acid are obtained.

HPLC titre:  $\geq$  99% performed under the following  
25 chromatographic conditions:

Column: Hibar<sup>R</sup> Lichrosphere RP18-5  $\mu$ m (Merck):  
250 mm length; 4 mm diameter.

Mobile phase: A - phosphate buffer pH 7.8;

30 B = 25% methanol in phosphate buffer pH 7.8 (v/v).

Flow rate: 1.2 ml/min; column temperature: 35°C; UV

254 nm detector.

Gradient: from 0 to 2 min B = 12%, from 2 to 20 min  
straight gradient up to B = 80% then  
maintenance for 3 min, then inverse  
5 gradient from 23 to 35 min up to B = 12%.

Following the same procedure the following free acid  
compounds have been obtained:

- (6S)-folinic acid with an O.P. higher than 99%,  
starting from calcium (6S)-folinate of Example 6.
- 10 - (6R)-folinic acid with an O.P. higher than 99%,  
starting from ethylenediamino (6R)-folinate of  
Example 6.

#### EXAMPLE 2

Preparation of ethylenediamino (6R,S)-folinate.

- 15 75 g of (6R,S)-folinic acid are dissolved in 750 ml of  
dimethylacetamide (DMAC). 10.5 g of ethylenediamine,  
dissolved in 400 ml of the same solvent, are added at a  
temperature of 5-35°C. The precipitated salt suspension  
is kept under stirring for about 1 h, at 5-25°C, then  
20 is filtered. After drying, 82 g of ethylenediamino  
(6R,S)-folinate are obtained.

HPLC titre: > 98.5% according to the chromatographic  
method of Example 1.

#### EXAMPLE 3

- 25 Diastereomer separation of ethylenediamino (6R,S)-  
folinate.

- 80 g of ethylenediamino (6R,S)-folinate are dissolved  
in 740 ml of water. 920 ml of DMAC are added under  
stirring at a temperature of 25-40°C. Then temperature  
30 is lowered to 5°C and the product crystallizes, under  
stirring, for 24 h. After that the precipitate is

filtered off and washed with ethanol. After drying, 40.2 g of ethylenediamino folinate are obtained, containing the 80% of (6R) diastereoisomer. The (6R) form content is determined by performing a chiral HPLC under the following conditions:

Column: length 250 mm; diameter 4 mm; manually fed with Chiral Si 300 BSA (SERVA), 5  $\mu$ m stationary phase.

Mobile phase: 0.25 M solution of  $\text{NaH}_2\text{PO}_4$  in water.

10 Flow rate: 1.0 ml/min; column temperature 40°C; detector: UV 280 nm (310 nm).

Mother liquors, after filtration, contain ethylenediamino (6S)-folinate with a 60% diastereomeric excess (determined with chiral HPLC under the above mentioned conditions).

Chromatographic conditions disclosed in Examples 1 and 3 are also used for controls and determinations in the remaining examples.

#### EXAMPLE 4

20 Ethylenediamino (6S)-folinate isolation and purification.

To the mother liquors derived from the crystallization described in Example 3 and mostly containing ethylenediamino (6S)-folinate, with a 60% diastereomeric excess, 170 ml of DMAC are added. The solution is kept under stirring for 48 h at 10°C. The resulting precipitate is filtered, washed with ethanol and dried. 15 g of ethylenediamino (6S)-folinate are obtained with an O.P. higher than 99% (chiral HPLC control was performed according to Example 3).

30 HPLC titre:  $\geq 98.5$ ;  $[\alpha]_D^{20} = -14.8^\circ$  ( $c = 2.1\% \text{ H}_2\text{O}$ ).

## EXAMPLE 5

Preparation of calcium (6S)-folinate from ethylenediamino (6S)-folinate.

To 10 g of ethylenediamino (6S)-folinate (obtained as disclosed in Example 4), dissolved into 100 ml of water, 10 g of calcium chloride and 400 ml of ethanol are added under stirring at 5-25°C. The separated precipitate is filtered, washed with aqueous ethanol (95%) and dried. Then, the product is dissolved again in water at pH 6-6.5, at 50-60°C. One part in weight of calcium chloride is added and pH is adjusted to 7 with NaOH 1N. The desired compound crystallizes at 0-25°C for 16 h.

The precipitated crystalline product is filtered, washed with cold water and aqueous ethanol and dried. 11 g of calcium (6S)-folinate containing about 20% of crystallization water are obtained with an O.P. higher than 99% (through chiral HPLC).

HPLC titre: > 99%;  $[\alpha]_D^{20} = -15^\circ$  (c= 1% H<sub>2</sub>O).

20

## EXAMPLE 6

Purification of ethylenediamino (6R)-folinate.

35 g of ethylenediamino (6R)-folinate (obtained as described in Example 3), with 80% O.P., are recrystallized from 500 ml of water/DMAC with a volumetric ratio of 1/1.25. Temperature and crystallization time are the following: 0-25°C and 48h. The crystallized product is filtered, washed with ethanol and dried. 25 g of ethylenediamino (6R)-folinate with an O.P. higher than 99% (chiral HPLC) are obtained.

30  $[\alpha]_D^{20} = +44.4^\circ$  (c=2.1% H<sub>2</sub>O).



**EXAMPLE 7**

Calcium (6S)-folinate isolation and purification from an ethylenediamino folinate mixture enriched in the (6S) isomer.

5 The mother liquors deriving from the crystallization described in Example 3 and mostly containing ethylenediamino (6S)-folinate with a 60% diastereoisomeric excess are extracted with 5 x 400 ml methylene chloride. To the resulting aqueous solution, 50 g of  
10 calcium chloride are added under stirring at 0-25°C, then pH is adjusted to 7 with NaOH 1 N and 250 ml of ethanol are added. The solid separated product is filtered, washed with aqueous ethanol and dried. The resulting product is recrystallized twice from water,  
15 at pH 7, at 0-25°C and in the presence of 3 parts in weight of calcium chloride and washing the filtered product every time, the first time with cold water and then with ethanol.

17 g of calcium (6S)-folinate are obtained with an O.P.  
20 higher than 99% (through chiral HPLC) and with about 20% crystallization water content.

HPLC titre: > 99%;  $[\alpha]_D^{20} = -15^\circ$  (c=1% H<sub>2</sub>O).

**EXAMPLE 8**

Piperazino (6R,S)-folinate preparation.

25 80 g of (6R,S)-folinic acid, prepared as described in Example 1, are dissolved in 950 ml of DMAC. 17.5 g of piperazine dissolved into 2000 ml of ethanol are added thereto under stirring at a temperature of 5-30°C. The resulting suspension is kept under stirring for 3 h at  
30 room temperature and then filtered. The wet product is washed with ethanol, and then dried. 90.5 g of

piperazino (6R,S)-folinate are obtained.

HPLC titre: > 99%.

#### EXAMPLE 9

Diastereomer separation of piperazino (6R,S)-folinate.

5 85 g of piperazino (6R,S)-folinate are suspended in  
3600 ml of a mixture water/DMAC (1:10.5 v/v).  
Temperature is raised to 35°C, until the solid compound  
is completely dissolved. Crystallization takes 3 days  
at 5°C, under stirring. The obtained precipitate is  
10 filtered, washed with ethanol and dried. 35 g of  
piperazino (6S)-folinate with an O.P. of  $\geq$  97% are  
recovered.

#### EXAMPLE 10

Piperazino (6S)-folinate purification.

15 10 g of piperazino (6S)-folinate, obtained as described  
in Example 9, are recrystallized from a mixture  
water/DMAC (1:10.5 v/v) following the procedure of  
Example 6. After filtering, washing and drying the  
crystalline product, 8 g of piperazino (6S)-folinate  
20 with an O.P. higher than 99% (chiral HPCL control) are  
obtained.

#### EXAMPLE 11

Piperazino (6R)-folinate isolation and purification.

To the mother liquors derived from the crystallization  
described in Example 9, mostly containing piperazino  
25 (6R)-folinate, 1800 ml of ethanol are added. Crystalli-  
zation takes place at 5°C, for 64 h. The precipitate is  
then filtered, washed with ethanol and dried. 30 g of  
piperazino (6R)-folinate, with an O.P. of  $\geq$  94% (chiral  
30 HPCL control) are recovered.

Said salt is then recrystallized from a mixture

water/DMAC/ethanol (1:10:5 v/v/v), following the procedure of Example 6, to give the desired product with an O.P. higher than 99%.

#### EXAMPLE 12

5 Preparation of calcium (6S)-folinate from piperazino (6S)-folinate.

Calcium (6S)-folinate with an O.P. higher than 99% is obtained in yield of 80% from the piperazino (6S)-folinate deriving from Example 10 following the  
10 procedure described in Example 5.

#### EXAMPLE 13

Preparation of 1,4-dimethylpiperazino (6R,S)-folinate  
80 g of (6R,S)-folinic acid, prepared as described in Example 1, are dissolved into 800 ml of DMAC. 21 g of  
15 1,4-dimethyl-piperazine, dissolved in 460 of DMAC, are added under stirring at a temperature of 5-25°C, then 700 ml of ethanol are added. The solution is kept under stirring for 5 days at 5°C to obtain a precipitate which is filtered, washed with ethanol and dried.

20 90 g of 1,4-dimethylpiperazine (6R,S)-folinate are obtained.  
(HPLC titre: > 99%).

#### EXAMPLE 14

Preparation of 1,3-diamino-2-hydroxy-propane (6R,S)-fo-  
25 linate.

80 g of (6R,S)-folinic acid, prepared as described in Example 1, are dissolved into 800 ml of DMAC. 16.8 g of 1,3-diamino-2-hydroxy-propane dissolved into 420 ml of DMAC are added under stirring at 5-25°C. The solution  
30 is kept under stirring for 1.5 h at the same temperature to obtain a precipitate which is filtered,

washed first with DMAC and then with ethanol, and then dried. 89 g of 1,3-diamino-2-hydroxy-propane (6R,S)-folinate are obtained (HPLC titre > 99%).

#### EXAMPLE 15

5 Partial separation of 1,3-diamino-2-hydroxy-propane (6R,S)-folinate diastereomers.

87 g of 1,3-diamino-2-hydroxy-propane (6R,S)-folinate are dissolved into 600 ml of water. 780 ml of DMAC are added under stirring and at a temperature of 25-40°C.

10 The product crystallizes at 5°C from the solution kept under stirring for 2 days, then is filtered. The wet product is washed with ethanol. After drying, 50 g of 1,3-diamino-2-hydroxy-propane folinate containing about 70% of diastereomer (6R) (chiral HPLC), are obtained.

15 In the residual crystallization mother liquors, the (6S) diastereomer is present in about 55% diastereomeric excess.

#### EXAMPLE 16

1,3-diamino-2-hydroxy-propane (6S)-folinate isolation and purification.

20 160 ml of DMAC are added to the mother liquors deriving from the fractional crystallization described in Example 15, and containing 1,3-diamino-2-hydroxy-propane (6S)-folinate, with a 55% diastereomeric excess.

25 The solution is kept under stirring for 30 h at 5°C to obtain a precipitate which is filtered and washed with ethanol. After drying, 20 g of 1,3-diamino-2-hydroxy-propane (6S)-folinate are obtained with 95% O.P. This product is recrystallized from 300 ml of

30 water/DMAC mixture in a volumetric ratio of 1/1.5 at a 0-25°C temperature. The resulting precipitate is

filtered and washed with ethanol. After drying, 16 g of 1,3-diamino-2-hydroxy-propane (6S)-folinate are obtained with an O.P. higher than 99% (determined through chiral HPLC), titre  $\geq$  98.5%.

CLAIMS

1. A process for the separation of the (6R) and (6S) diastereomers of folinic acid and the recovery thereof in free or salified form of desired high optical purity, which process comprises the steps of:
- 5 in free or salified form of desired high optical purity, which process comprises the steps of:
- a) reacting racemic folinic acid, free or as a salt with an alkaline-earth metal, in a liquid medium containing at least one component selected from
- 10 dipolar aprotic organic solvents, water and water-soluble protic organic solvents, with an aliphatic straight or cyclic amine having at least two amino groups connected together through at least one substituted or unsubstituted C<sub>2</sub>-C<sub>3</sub> hydrocarbon chain, to give the corresponding racemic folinic diamino-salt;
- 15 diamino-salt;
- b) crystallizing said diamino-salt from its solution in a mixture of water and a water-soluble dipolar aprotic organic solvent, optionally containing an
- 20 organic water-soluble protic organic solvent, wherein a first solid which separates upon cooling contains a major portion of a first one of the said (6R) or (6S) diastereomers, the major portion of the other second isomer remaining in the crystallization mother-liquors;
- 25 zation mother-liquors;
- c) purifying by recrystallizing once or more times the isomer-enriched solid form step (b), from the same solvent mixture used in this step, until the first diastereomeric diamino-salt is obtained in a
- 30 desired optical purity, and optionally converting this salt into the corresponding alkaline-earth

metal salt;

- 5 d) isolating the solid remaining dissolved in the mother-liquors from step (b), through precipitation by diluting the solution with a dipolar aprotic solvent and/or a protic organic solvent, wherein the solid that is obtained mainly contains the second isomer, and recrystallizing this solid as recited in step c), or treating said mother-liquors, after evaporation of the organic solvents, with an excess of water-soluble alkaline-earth metal mineral salts and recrystallizing once or more times the obtained alkaline-earth metal salt of said second isomer of folinic acid with water until the desired optical purity is reached.
- 10 2. The process of claim 1, in which the amine used in step a) is selected from straight, alicyclic or heterocyclic di- or poly amines optically active or not and the nitrogen atoms of which are unsubstituted or substituted by organic groups.
- 15 3. The process of claim 1, in which in step (a) an alkaline-earth metal salt of racemic folinic acid is used, wherein said alkaline-earth metal cation is insolubilized by reaction with a suitable anion and filtered off.
- 20 4. The process of claim 1, in which the racemic diamino-salt of folinic acid is isolated in step (a) by precipitation or crystallization, treating the reaction mixture with a water-soluble protic organic solvent.
- 25 5. The process of claim 1, in which the desired optical purity degree for both isomers which result from crystallization in steps (c) or (d) is 99% or more.
- 30

6. The method of claim 1 for the separation and the purification of the two diastereomeric forms, (6R) and (6S), of folinic acid and/or its salts with alkaline-earth metal ions or with at least dibasic organic amines, up to an optical purity (O.P.) level higher than 99%, starting from a racemic mixture of the same, this process comprising the following steps:

- a) preparing the diamino salts of said racemic mixture by reacting racemic free folinic acid, dissolved in a dipolar aprotic organic solvent or in a mixture of the same with a protic organic solvent, or suspended in water, with straight, cyclic or heterocyclic, substituted or unsubstituted, racemic or chiral, aliphatic di- or polyamines, which contain at least two amino groups linked to one another by at least one substituted or unsubstituted hydrocarbon chain of at least 2 or 3 C atoms,
- or preparing said diamino salts by reacting an aqueous solution of a racemic alkaline-earth metal folinate with said amines and eliminating said metal ions by formation of insoluble compounds of the same and filtration thereof, wherein the obtained racemic mixture of said diamino salts crystallizes from the reaction medium or is precipitated from the same by adding a water-soluble protic organic solvent or by diluting said reaction medium in said water-soluble protic organic solvent,
- b) crystallizing said precipitated racemic mixture form a solution thereof in a solvent made of water



- and a water-soluble dipolar aprotic organic solvent optionally containing a protic organic solvent, wherein the solid that separates upon crystallization contains a major portion of one of the two (6R) or (6S) diastereomeric forms of the diamino salt, this form being less soluble than the other one, which remains largely dissolved in crystallization mother liquors,
- 5                   c) recrystallizing once or more times the isomer-enriched solid obtained under step b), from the same kind of solvent used in this step, to give the desired diamino (6R) or (6S)-folinate with an O.P. higher than 99%, wherein said optically pure diamino folinate can be subsequently transformed
- 10                   into the corresponding alkaline-earth metal salts of the same optical purity by reaction with an excess of water-soluble alkaline-earth metal mineral salts in aqueous medium,
- 15                   d) precipitating the other isomer-enriched diamino salt from crystallization mother liquors, obtained under step b), by dilution with a dipolar aprotic solvent and/or a protic organic solvent, collecting the solid thereof and recrystallizing it once or more times under the same conditions as in step c),
- 20                   wherein said other stereoisomeric form of diamino-folate is obtained with an O.P. higher than 99%, the corresponding alkaline-earth metal salts being then optionally obtained with the same optical purity by reaction with an excess of water-soluble alkaline-earth metal mineral salts,
- 25                   or directly treating said mother liquors of step
- 30

b), after elimination of any organic solvent, with an excess of water-soluble alkaline-earth metals mineral salt, wherein the obtained isomer-enriched alkaline-earth metal folinates are recrystallized once or more times from water to give the corresponding metal folinates with an O.P. higher than 99%.

7. The method of claim 6, wherein, during step a), the reaction of racemic folinic acid with said amines is performed in a dipolar aprotic organic solvent, or in a mixture of the same with a protic organic solvent, or in water, in suspension phase, by subsequently diluting the resulting solution with or in a water-soluble organic protic solvent.

8. The method of claim 6, wherein, during step a), the reaction of said racemic alkaline-earth metal folinates with said amines is performed in water in the presence of inorganic or organic anions, which forms water-insoluble derivatives with said alkaline-earth metal cations, being said resulting insoluble compounds preferably sulphates or oxalates.

9. The method of claim 6, wherein crystallizations under steps b) and c) are performed in a solvent mixture made of water and a water-soluble dipolar aprotic organic solvent, wherein said components range, in a volumetric ratio, from 1:0.5 (v/v) to 1:20 (v/v), preferably from 1:1 (v/v) to 1:15 (v/v), or said crystallizations are performed in a solvent containing said water/water-soluble dipolar aprotic organic solvent mixture further added with a protic organic solvent in amounts up to 70% in volume, preferably up

to 50% (v/v).

10. The method of claim 6, wherein the weight/volume ratio between said racemic diamino folinate salts and the solvent mixture used for crystallization of the same, according to step b), ranges from 1:2 (w/v) to 1:60 (w/v), preferably from 1:4 (w/v) to 1:45 (w/v).

11. The method of claim 6, wherein crystallization of said salts of folinic acid is performed according to steps b) or c), by dissolving them in said solvent mixtures at a temperature ranging from 25 to 70°C, preferably from 30 to 50°C and then by gradually cooling the resulting solution to 25-0°C, preferably to 20-0°C.

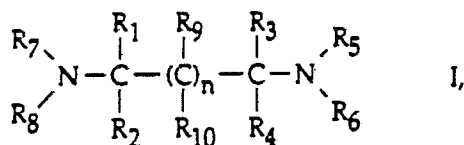
12. The method of claim 6, wherein the optically pure (6R) or (6S) diamino folinates, obtained under step c), are transformed into the corresponding alkaline-earth metal salts of the same optical purity in water, in presence of water-soluble mineral salts of said metals in a weight ratio (w/w) ranging from 0.2 to 5, preferably 2 to 4, parts of said metal salt per part of said diamino salt, at a temperature ranging from 0 to 35°C, preferably 5 to 25°C, by maintaining pH between 6 and 7, preferably by adding 1N sodium hydroxide.

13. The method of claim 6, wherein preferred cations of alkaline-earth metal are selected from  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Sr}^{2+}$ ,  $\text{Ba}^{2+}$ .

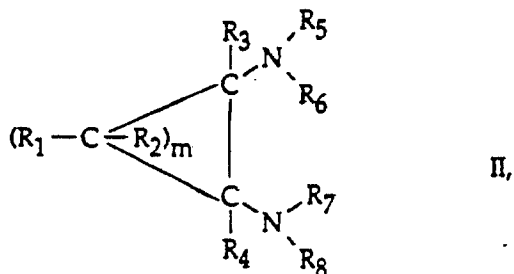
14. The method of claim 13, wherein the preferred metal ion is  $\text{Ca}^{2+}$ .

15. The method of claim 1, wherein the preferred amines, used in stoichiometric amount or in a molar excess not higher than 20%, are selected among diamines

of general formulae from I to III

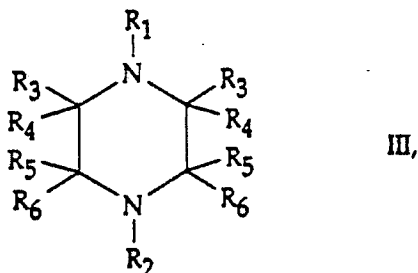


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wherein:

R<sub>1</sub> to R<sub>8</sub> which are the same or different, are H or straight or branched C<sub>1-6</sub> alkyl groups, which can be substituted by 1-4 OH groups and/or by 1-4 alkoxy or hydroxyalkoxy groups,

20

R<sub>9</sub> to R<sub>10</sub> which are the same or different, are R<sub>1</sub> or OH,

n 0-6,

25 m 2-8.

or they are selected from straight or macrocyclic polyamines comprising from 3 to 6 N atoms.

16. The method of claim 15, wherein the preferred amines are selected from: ethylenediamine, 1,2-diaminopropane, 1,3-diaminopropane, 1,3-diamino-2-hydroxypropane, (cis)-1,2-diamino-cyclohexane, (trans)-1,2-

30

diamino-cyclohexane, piperazine, 2-methyl-piperazine, 1,4-dimethyl-piperazine, 2,5-dimethyl-piperazine, 1,4,7,10-tetraazacyclododecane, derivatives and lower and higher homologous thereof.

5 17. The method of claim 6, wherein the preferred dipolar aprotic solvents are selected from: dimethylformamide, dimethylacetamide, dimethylsulphoxide, N-methyl-pyrrolidone, hexamethylphosphoramide.

10 18. The method of claim 6, wherein the preferred protic organic solvents are selected from: methanol, ethanol, n-propanol, isopropanol, ethylene glycol, 1,2-propylene glycol, 1,3-propylene glycol, formamide, N-methyl-formamide.

15 19. The method of claim 6, wherein the optically pure salts of folinic acid, obtained under steps c) or d), are dissolved in water and the resulting aqueous solutions are acidified by adding a mineral acid to a pH value of 1-3 at a temperature ranging from 0 to 25°C, wherein the corresponding (6R) or (6S) isomers of  
20 free folinic acid precipitate with an O.P. higher than 99%.

20. The method of claim 12, wherein said water-soluble mineral salts of alkaline-earth metals are selected from chlorides and nitrates.

25 21. Diamino (6R,S)-folinates wherein the salifying diamine is selected from: ethylenediamine, 1,2-diaminopropane, 1,3-diaminopropane, 1,3-diamino-2-hydroxypropane, (cis)-1,2-diamino-cyclohexane, (trans)-1,2-diamino-cyclohexane, piperazine, 2-methyl-piperazine, 1,4-dimethyl-piperazine, 2,5-dimethyl-piperazine.  
30

22. Diamino (6R)-folinates with an O.P. higher than

99%, wherein the salifying diamine is selected from:  
ethylenediamine, 1,2-diamino-propane, 1,3-diamino-  
propane, 1,3-diamino-2-hydroxy-propane, (cis)-1,2-  
diamino-cyclohexane, (trans)-1,2-diamino-cyclohexane,  
5 piperazine, 2-methyl-piperazine, 1,4-dimethyl-  
piperazine, 2,5-dimethyl-piperazine.

23. Diamino (6S)-folinates with an O.P. higher than  
99%, wherein the salifying diamine is selected from:  
10 ethylenediamine, 1,2-diamino-propane, 1,3-diamino-  
propane, 1,3-diamino-2-hydroxy-propane, (cis)-1,2-  
diamino-cyclohexane, (trans)-1,2-diamino-cyclohexane,  
piperazine, 2-methyl-piperazine, 1,4-dimethyl-  
piperazine, 2,5-dimethyl-piperazine.

24. A diamino-folate selected from:  
15 Ethylenediamino (6R,S)-folinate;  
Piperazino (6R,S)-folinate;  
1,4-dimethyl-piperazino (6R,S)-folinate;  
1,3-diamino-2-hydroxy-propyl (6R,S)-folinate;  
Ethylenediamino (6R)-folinate;  
20 Ethylenediamino (6S)-folinate;  
Piperazino (6R)-folinate;  
Piperazino (6S)-folinate;  
1,4-dimethyl-piperazino (6R)-folinate;  
1,4-dimethyl-piperazino (6S)-folinate;  
25 1,3-diamino-2-hydroxy-propyl (6R)-folinate;  
1,3-diamino-2-hydroxy-propyl (6S)-folinate.

## INTERNATIONAL SEARCH REPORT

PCT/EP 93/00361

International Application No

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (If several classification symbols apply, indicate all) <sup>6</sup>		
According to International Patent Classification (IPC) or to both National Classification and IPC		
Int.Cl. 5 C07D475/04		
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched <sup>7</sup>		
Classification System	Classification Symbols	
Int.Cl. 5	C07D	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched <sup>8</sup>		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup></b>		
Category <sup>10</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
A	EP,A,0 266 042 (UNIVERSITY OF STRATHCLYDE) 4 May 1988 *Document*	1
A	EP,A,0 348 641 (EPROVA) 3 January 1990 *Document*	1
A	EP,A,0 356 934 (KYOWA HAKKO KOGYO) 7 March 1990 *Document*	1
A	EP,A,0 367 902 (EPROVA) 16 May 1990 *Document*	1
A	EP,A,0 432 441 (AMERICAN CYANAMID COMPANY) 19 June 1991 *Document*	1
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<p><sup>10</sup> Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&amp;" document member of the same patent family</p>		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
27 MAY 1993	- 7. 06. 93	
International Searching Authority	Signature of Authorized Officer	
EUROPEAN PATENT OFFICE	LUYTEN H.W.	

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category <sup>a</sup>	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
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**ANNEX TO THE INTERNATIONAL SEARCH REPORT  
ON INTERNATIONAL PATENT APPLICATION NO.**

EP 9300361  
SA 70918

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27/05/93

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